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OF COMPLEX SYSTEMS

Effect of the Nitric Oxide Synthesis Inhibitor L-NAME on the Isolated Rat Heart after Hypokinesia

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Abstract—The effects of a non-selective nitric oxide synthase inhibitor L-NAME on the functional parameters of the isolated rat heart after a 30-day period of hypokinesia were studied. Electron paramagnetic resonance spectroscopy was employed in the analysis of a role for L-NAME in the intensity of nitric oxide production in rat heart tissues. The level of nitric oxide synthesis was assessed by the intensity of the signal belonging to the $(DETC)_2$–Fe^{2+}–NO complex. It was found that L-NAME decreased nitric oxide production on average by 69%. The Langendorff isolated perfused heart was used to evaluate cardiac activity, and the following parameters were measured: pressure generated by the left ventricle, heart rate, and coronary flow. Addition of the nitric oxide synthesis inhibitor L-NAME induced an increase in the inotropic function and normalization of the heart rate.

Keywords: isolated heart, hypokinesia, nitric oxide, electron paramagnetic resonance, pressure generated by the left ventricle, heart rate, coronary flow

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The levels of daily physical activity in modern humans are decreasing because of urbanization, automation and mechanization of labor due to the technological progress, as well as in the context of severe epidemiologic situation and self-isolation. Hypokinesia (restriction of motor activity) leads to morphofunctional shifts in the major physiological systems to pathological states, the severity of which depends on the duration and degree of motor restriction [1–6]. Diminished afferent input lowers the tone of the central nervous system and changes the structure and function of synapses, as well as the muscle trophics [6]. Restriction of mobility has its effect on almost all systems of the body. The following changes have been identified in the state of the cardiovascular system: changes in the contractile function of the heart muscle, weakening of the myocardium and the coronary vessels, decrease in the energy potential of the heart, and reduction in the minute volume [2, 7]. Furthermore, hypokinesia suppresses the body's defense systems and reduces the resistance of the circulatory system to damaging factors, which can lead to cardiovascular diseases [8]. Increasing motor restriction augments the risk of cardiovascular diseases and thus becomes a serious threat to health.

Many studies have shown that hypokinesia affects the activation of free radicals in the body and the free-radical damage to the heart induced by stress factors. Under conditions of prolonged motor restriction, activation of the nitric oxide (NO) system can be considered as a component of the stress response [9, 10]. A huge amount of experimental and clinical material has been accumulated on the physiological and pathophysiological effects of nitric oxide, as well as on the conjugation of its effects with superoxide radical in the realization of oxidative stress [11, 12].

NO was for initially identified as an inflammation marker and a regulator of vascular tone. However, subsequent studies showed that this molecule has a much broader regulatory effects. Since the 1980s, NO is recognized as an important endothelium-derived molecule with a crucial significance for the maintenance of cardiovascular homeostasis. NO is known as a major

Abbreviations: NO, nitric oxide; NOS, NO synthase; cGMP, cyclic guanosine monophosphate; AR, adrenergic receptor; CR, cholinergic receptor; L-NAME, NG-nitro-L-arginine methyl ester; EPR, electron paramagnetic resonance; DETC, diethyldithiocarbamate; PGLV, pressure generated by the left ventricle; HR, heart rate.

biological mediator involved in various physiological and pathophysiological processes [13–20]. In biological systems, NO is generated as a result of L-arginine oxidation mediated by NO synthase (NOS). Conventionally, all NOS isoforms were classified into constitutive (cNOS) and inducible (iNOS) ones. In 1990, the neuronal form of the enzyme (nNOS) was isolated from the rat brain; subsequently, inducible NOS (iNOS) was found in immune system cells (macrophages), and endothelial NOS (eNOS) was discovered in the endothelium. A further NOS isoform is present in mitochondria (mt-NOS); it regulates cellular respiration. It currently remains unclear whether mt-NOS represents a separate NOS isoform or iNOS modified in the course of translation or post-translationally [21, 22].

NO does not require receptors and easily diffuses through cell membranes to enter the neighboring cells. The key target of NO is intracellular soluble guanylate cyclase: it is activated to produce cyclic guanosine monophosphate (cGMP), which mediates all effects of NO. In particular, cGMP decreases the level of free Ca^{2+} and activates myosin light chain kinase, causing relaxation of vascular smooth muscles. Impairment of this mechanism leads to hypertension.

The role of NO in the cardiovascular system is well known and involves regulation of multiple cellular processes. Cardiovascular disorders are associated with enhanced or diminished NO production. Deregulation of NO bioavailability contributes to development of various heart diseases, and intense research is aimed at elucidating the underlying processes. An accumulating body of data implicates impaired NO metabolism and/or its abnormal action in the development of ischemic heart disease and heart failure [23]. Under pathological conditions, NOS can be disassembled, which results in production of reactive oxygen species (ROS) instead of physiological NO production [24]. NO is synthesized by all cell types of the myocardial tissue and regulates heart function via vascular-dependent and -independent effects. The first group includes regulation of coronary vessel tone, blood clotting, proliferative and inflammatory processes, as well as maintenance of tissue regeneration. The second group explains direct effects of NO on certain aspects of cardiomyocyte contractility, from the fine regulation of excitation–contraction coupling to the modulation (presynaptic and postsynaptic) of sympathetic and parasympathetic influences. Nitric oxide has a pronounced effect on the heart, causing a decrease in heart rate and stroke volume, as well as an increase in PQ interval duration and blood ejection period. Its action is realized through different subtypes of adrenergic (AR) and cholinergic (CR) receptors. Negative inotropic and chronotropic effects of nitric oxide on the heart are realized through modulation of adrenergic and cholinergic influences mediated by β_1-AR, β_2-AR, α_1-AR, and m-CR [25]. It is known that α_2-AR activation significantly stimulates endothelial NOS, thus causing an increase in the NO level [26]. All α_2-AR subtypes (A, B, and C) are responsible for NO synthesis and decrease of the intracellular calcium ion level in the cytosol mediated by Ca^{2+}-ATPase of the sarcoplasmic reticulum [27]. NO and cGMP are the central intracellular messengers of α_2-AR signaling in ventricular myocytes [28].

NO is the key modulator of mechanical forces in cardiomyocytes. NO synthesized and released by endothelial cells mediates various effects, such as vascular tone, hemostasis, blood pressure, and vascular remodeling. NO can also activate the Na–K pump of the outer cell membrane, causing its hyperpolarization. It is this mechanism that leads to vasodilation with an increase in blood flow and tension (e.g., pulse) of the vascular wall. NO supports active vasodilation, regulates blood flow, and controls basal arterial pressure.

Various L-arginine analogs that act as competitive NOS inhibitors can suppress NO synthesis. NG-nitro-L-arginine methyl ester (L-NAME) competes with L-arginine for the binding site of the enzyme and thus blocks the enzyme activity. Inhibition of NO synthases due to chronic administration of L-NAME leads to a decrease in the heart rate, stroke volume, minute cardiac output, and flow rate of blood ejection, and on the other hand, increases the ejection time, arterial pressure, peripheral vascular resistance, power of heart contraction, and energy expenditure for transportation of 1 L blood. It was shown that L-NAME enhances the cholinergic effects on the heart rate (HR). The decrease in the stroke volume in rats is due to a decline in the regulatory effects mediated by α_1-AR and increased role of m_2-CR. These effects of blocked NO synthesis may be related to inhibition of its presumed ability to diminish myofilament sensitivity to Ca^{2+}. The decrease in the inotropic function of the heart may also be due to elimination of the inhibitory effect of NO on the tone of vascular muscles and accordingly to an increase in their tonic contraction and a dramatic decrease in the coronary flow [25].

Researchers indicate that hypokinesia is associated with a significant increase in the NO levels in the heart. NO is involved in adaptation to stress conditions. It was shown that after restriction of motor activity for 30 days, NO production in heart tissue increased 1.5-fold in comparison to animals kept under vivarium standard conditions (three or four animals per cell) [29]. The increased rate of NO production during hypokinesia indicates that NO production in the body strongly depends on the character of motor activity. NO is an active metabolic player, and a dramatic change in its generation can impair the functional activity of many biosystems. The published data

show that mobility restriction leads to significant changes in the cardiovascular system, blood flow, and oxygen supply; it can be assumed that some of these changes are caused by an increase in NO production in these structures. Therefore, one of the promising targets of therapeutic interventions in hypokinesia is the NO system. Considering that NO production increases during motor restriction, quantitative evaluation of NO content in the heart under the conditions of NO synthase inhibition represents a topical issue, and so is investigation of the effects of L-NAME on the functional parameters of the isolated heart in hypokinesia.

MATERIALS AND METHODS

The experiment was carried out in outbred white laboratory rats that were kept under conditions of restricted motor activity (hypokinesia) for 30 days. The animals were divided in two groups: animals of both groups were subjected to 30 days of hypokinesia, and animals of group 2 were additionally administered L-NAME, a nonselective NOS inhibitor. Each group included 15 animals. To model hypokinesia, rats were placed into individual pen-case-like cages of polyacrylate for 30 days. The duration of exposure to hypokinetic conditions was 1 h on day 1, 2 h on days 2 and 3, and was subsequently increased by 2 h every two days. This protocol was described in detail in our previous work [30]. A distinguishing feature of this hypokinesia model (with horizontal body position) is a diminished effect of the immobilization stress, which is achieved by gradually increasing the time of exposure to the conditions of restricted mobility. In contrast to the antiorthostatic model (which involves hanging the rats by the tail), our model also eliminates the additional effect of the hydrostatic blood pressure change.

Nonselective NOS inhibitor, L-NAME, was administered intraperitoneally in a dose of 10 mg/kg 60 min prior to decapitation. The animals were anesthetized with uretane in a dose of 1200 mg/kg body weight administered intraperitoneally.

Detection and quantitative determination of NO in heart specimens was performed using electron paramagnetic resonance (EPR) spectroscopy with spin trapping. The method is based on capturing NO radicals by a spin trap, namely, the Fe^{2+} complex with diethylthiocarbamate (DETC), with formation of a stable triple complex ($DETC_2$–Fe^{2+}–NO. To induce the formation of these complexes, the animals were administered sodium DETC solution intraperitoneally (a dose of 500 mg/kg in 2.5 mL water) and iron citrate solution intramuscularly (iron (II) sulfate ($FeSO_4 \cdot 7H_2O$, Sigma, United States) in a dose of 37.5 mg/kg + sodium citrate, 187.5 mg/kg). The method was described in detail in our previous works [31, 32]. The nitrogen oxide trap was injected 30 min before autopsy. The DETC–Fe(II) complex interacts with NO, producing the stable radical $(DETC)_2$–Fe^{2+}–NO. This complex is paramagnetic ($S_{Fe} = 1/2$; $I_N = 3/2$) and can be detected by EPR spectroscopy [32]. It is characterized by an easily recognizable EPR spectrum with a *g* factor of 2.035 and a triplet ultrathin structure. NO content was assessed by the intensity of the characteristic EPR signal produced by the $(DETC)_2$–Fe^{2+}–NO complex. The signals were compared based on their integral intensities, since the integral intensity of the EPR signal is directly proportional to the concentration of the paramagnetic complex [32]. In 30 min after drug administration, urethane-anesthetized rats were fixed on the operating table, opened, and the extracted organs were quickly dried and frozen in liquid nitrogen in capillaries for measurements. EPR spectra of the preparations were recorded at 77 K using an ER-200E-SRC spectrometer in X-band (Bruker, United States) with an ER 4112HV temperature control unit. The following parameters remained constant in all experiments: microwave power, 30 mW; modulation, 5 Gs; magnification, 4·104; time constant, 100 ms; recording time, 50 s; number of signals, 8. The spectra were accumulated and recorded using the computer of an Aspect 3000 spectrometer (Bruker). The prepared samples cut to fit the measurement cell were weighted immediately before spectroscopy; the weight of the samples was ~100 mg. Amplitudes of the EPR spectra were normalized by the weight of the sample and by the amplitude of the standard sample signal (the method of EPR signal measurement was described in detail previously in [33, 34]).

The Langendorff isolated heart method was used to analyze the activity of the isolated heart (as described in detail in our previous work [35]). In anesthetized rats, the chest was opened, and the heart was extracted and placed in cold Krebs–Henseleit working solution (pH 7.4). The heart was fixed on the cannula of the Langendorff unit and perfused with the working solution. The solution was saturated with oxygen, and the temperature was maintained at 37°C. Ventricular pressure was measured using a latex balloon that was inserted into the left ventricle.

The first 15 min after the beginning of perfusion were left for adaptation of the heart, and then the heart function parameters were recorded for 20 min. The assessed parameters were the pressure generated by the left ventricle (PGLV), the heart rate, and the coronary flow. The functional parameters of the isolated heart of rats that had been administered L-NAME were evaluated in proportion to the same parameters in rats of group 1. The values of the heart function parameters in rats of the control group were taken for 100%. Changes in the contractile force were assessed by the pressure generated by the left ventricle, and coronary flow was measured as the volume of the solution passing through the blood vessels of the heart. PGLV was

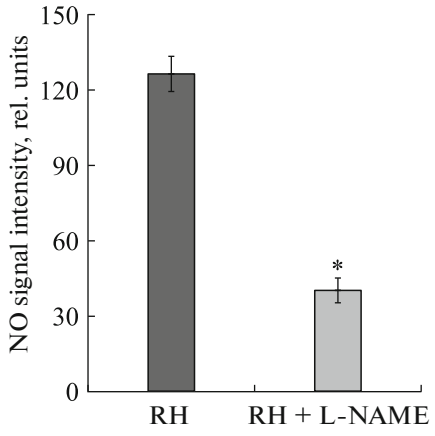

Fig. 1. Changes in the levels of NO-containing paramagnetic complex $(DETC)_2$–Fe^{2+}–NO in the heart tissue of rats with hypokinesia (RH) and after administration of L-NAME, a nonselective NOS inhibitor. *Y* axis, integral intensity of the EPR spectrum, rel. units. *Significance of difference from the control group, $P < 0.05$.

measured in mm Hg, heart rate, in beats per minute (bpm), and coronary flow, in mL/min. Ex vivo experiments in the isolated heart specimens were carried out using the Power Lab 8/35 equipment (ADinstruments, New Zealand); the data were recorded using the LabChart Pro software. Statistical analysis was performed using MS Excel; significance of the results was assessed with the Student's *t*-test and the Mann–Whitney U-test. Data for statistical analysis were presented as the mean and the standard error of the mean ($M \pm SEM$). Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Activation of the NO system is one of the mechanisms serving to prevent stress-induced damage. Exposure to 30 days of hypokinesia causes activation of local stress-limiting systems, including the system of NO generation. Previously, we showed that prolonged hypokinesia causes a significant increase in NO content in rat organs [36, 37]. Considering that NO production increases under these conditions, application of a NO blocker to prevent possible pathological changes related to the NO system metabolism in hypokinesia seems a relevant approach. On the other hand, clinical and experimental studies found that application of NO donors and NOS inhibitors was not accompanied by changes in the nitric oxide content in the heart tissues, even though it is of ultimate importance, since these compounds can have their own effects unrelated to those of nitric oxide. Dynamics of NO content in the heart under conditions of NOS inhibition can be assessed using EPR spectroscopy.

In this work, we used EPR spectroscopy to analyze quantitative changes in NO production in the heart tissue of rats exposed to hypokinesia with preliminary administration of L-NAME, a nonselective NOS inhibitor. NO production was evaluated by the intensity of the EPR signal corresponding to the $(DETC)_2$–Fe^{2+}–NO complex. It was found that the level of $(DETC)_2$–Fe^{2+}–NO in rats after 30 days of hypokinesia that were administered L-NAME, a nonselective NOS inhibitor, prior to the experiment (group 2) was on the average 69% lower than in the animals of group 1 ($P < 0.05$) (Fig. 1). The results of EPR spectroscopy confirmed that L-NAME actually decreased NO production in the heart of animals of group 2.

In the next series of experiments, heart function parameters in adult rats after 30 days of hypokinesia were studied using a Langendorff apparatus (Fig. 2). In rats of group 1, the coronary flow was 10.33 ± 0.51 mL/min, while in group 2, it was 6.84 ± 1.16 mL/min ($P < 0.05$). In rats after 30 days of hypokinesia, PGLV was significantly lower than normal and constituted 43.77 ± 3.16 mm Hg ($P < 0.05$); in animals of group 2 (who were administered L-NAME, a nonselective NOS inhibitor, after similar exposure to hypokinesia), PGLV was restored back to normal and differed significantly from the level of group 1 (mean, 67.76 ± 11.18 mm Hg; $P < 0.05$). Previously, it was shown that L-NAME affected arterial pressure: under conditions of unrestricted mobility, it caused an increase in the mean blood pressure by 25% in female rats and HR decrease by 18% [38]. In our experiments in hypokinetic rats, inhibition of NO synthases led to recovery of PGLV levels; this fact indicates that NOS inhibition has a positive inotropic effect in hypokinesia. In rats of group 1, HR values were elevated (188.42 ± 5.33 bpm), in agreement with the classic notion that long-term restriction of motor activity leads to tachycardia. In rats of group 2, this value was significantly lower and constituted 168.05 ± 11.55 bpm ($P < 0.05$). Therefore, inhibition of NO synthases in rats with hypokinesia results in HR normalization.

Analysis of the previously published data suggests that hypokinesia has a negative effect on the contractility of the myocardium, which modifies the systolic phase of the heart cycle (the period of tension increases, and the ejection period decreases). This is consistent with the results of our previous studies, which showed that restriction of motor activity led to an increase in HR together with a decrease in the force of contraction and in the coronary circulation [39]. Data presented in this work showed that after administration of L-NAME, a nonselective NOS inhibitor, these parameters exhibited an opposite dynamics: the inotropic function increased and the heart rate normalized. Thus, inhibition of the NOS system by L-NAME led to normalization of the functional parameters of the isolated heart after hypokinesia.

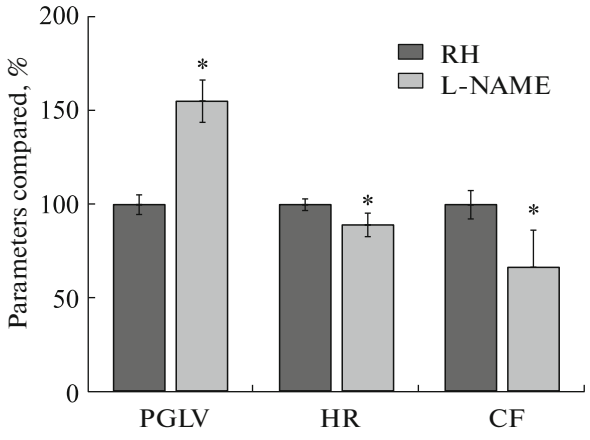

Fig. 2. Comparison of functional parameters of the isolated heart of rats with hypokinesia that were or were not administered L-NAME, a nonselective NOS inhibitor. RH, rats with hypokinesia; PGLV, pressure generated by the left ventricle; HR, heart rate; CF, coronary flow. Significance of differences in comparison to the control values: * $P < 0.05$.

These results contribute to our understanding of the role of nitric oxide and NO synthases in the cardiovascular system activity of rats exposed to stress conditions.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted in agreement with the principles of the Basel Declaration and according to the recommendations of the Bioethics Committee of the Kazan State University.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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